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# Trans-cis proline switches in a pentameric ligand-gated ion channel: how they are affected by and how they affect the biomolecular environment

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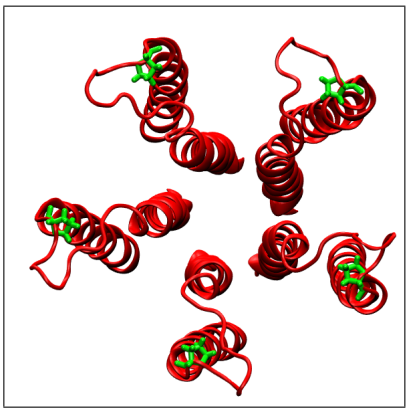
## Abstract

Pentameric ligand-gated ion channels (pLGICs) are important neuroreceptors, embedded in neuronal membranes, that mediate fast synaptic transmission. The molecular details of their working mechanisms have still to be fully unravelled due to their complexity and limited structural information available. Here we focus on a potential molecular switch in a prototypical pLGIC, the serotonin-activated 5-$HT_3$ receptor, consisting of the *trans-cis* isomerization of a proline at the interface between the extracellular and transmembrane domain. Mutagenesis electrophysiology experiments previously showed that if such isomerization could not take place, the channel would not open, but the hypothetical role of this mechanism as key to channel gating is still debated. We investigate this switch within the receptor with molecular dynamics and enhanced sampling simulations. We analyze how the isomerization free energy landscape is affected by the receptor environment in comparison to simplified models. Moreover, we reveal how the isomerization in turn affects the structural and electrostatic properties of the receptor at the extracellular-transmembrane domain interface, e.g. by tuning the ion selectivity filter.

## Graphical TOC Entry



## Keywords

Pentameric ligand-gated ion channels, 5-$HT_3$ receptor, molecular dynamics, free energy calculations, metadynamics, proline, *trans-cis* isomerization.

Pentameric ligand-gated ion channels (pLGICs) are important pharmaceutical targets for neurological disorders due to their role in fast synaptic transmission. They are embedded in the membrane of nerve cells and share common structural and functional features: they are composed of five subunits arranged around an ion-permeable pore, with an extracellular domain (ECD), a transmembrane domain (TMD) including the ion channel and, often, an intracellular domain (ICD). The opening of the TMD pore, which allows the ion exchange between the cytosol and the extracellular environment, is correlated to the binding of neurotransmitters at the interface between subunits in the ECD.[1,2] How the activation signal is rapidly transmitted from the ligand binding sites to the pore region that is tens of Å apart is still far from being understood. In addition to the intrinsic complexity of these receptors, limited structural information is available, although an increasing number of X-ray and cryo-electon microscopy (EM) fairly complete structures with good resolution are recently becoming available (see e.g. Refs.[3–16]).

The 5-hydroxytryptamine type 3 receptor (5-$HT_3$R) is a prototypical pLGIC, activated by the binding of serotonin (5-hydroxytryptamine, 5-HT).[17] It has been extensively studied experimentally because of the importance and versatility of functions it regulates: it is linked to neurological disorders such as schizophrenia and drug abuse, and is the target of antinausea drugs to alleviate side effects of anesthetics and chemotherapy.[18] In 2014, an X-ray structure of a mouse homo-pentameric 5-$HT_3$R has been resolved with a 3.5 Å resolution,[8] providing the unprecedented opportunity to build reliable models for atomistic simulations, without resorting to approximate homology modelling procedures. This structure was resolved in complex with antibodies and in the absence of ligands in the orthostetic sites and was proposed to be in a non-conductive closed or inactive state. This was supported by the channel profile reported both in the original work[8] and in subsequent studies.[19] New structures of the mouse 5-$HT_3$R have been determined more recently, such as a model by cryo-electron tomography in lipid vesicles at 12 Å resolution[11] and a cryo-EM apo-state in the closed conformation at 4.3 Å resolution.[14] The latter structure is similar, although with some differences, to the X-ray structure used to build the model investigated here, which is twisted counterclockwise with respect to the pLGIC putative closed conformations and thus appears to lay further along the expected pathway from closed to open structures.[14] Very recently a few cryo-EM complexes with agonists, antagonists and allosteric ligands interpreted in different non-conductive and conductive forms with resolution ranging from 3.2 to 4.5 Å have also been released,[15,16] providing a wealth of information for future studies. Unambiguously matching experimental structures to functional states is extremely challenging due to a variety of factors, including limited resolution, influence of detergent and/or nano-bodies, crystal packing and receptor engineering.[16]

As shown in Fig. 1 a), the ECD of 5-$HT_3$R is mainly composed of $\beta$-sheets, while the TMD of each subunit comprises four interconnected $\alpha$ helices (named M1-M4). Although topologically distinct, the ECD and TMD are coupled and can interact through a number of flexible interface loops, which are expected to play a major role in the transmission of the activation signal from the orthosteric binding sites to the pore-lining helices of the TMD. Relevant loops highlighted in Fig. 1 a) are the Cys-loop, which gives the name to the homonym pLGIC superfamily, the $\beta$1-$\beta$2 loop, the $\beta$8-$\beta$9 loop (also known as F loop) in the ECD and the M2-M3 loop in the TMD.

The M2-M3 loop, shown in Fig. 1 b), connects the M3 helix, which is partially exposed to the membrane, and the M2 helix, which lines the ion-permeable pore. A ring of negatively charged Asp271 residues at the top of the M2 helices acts as a sort of selectivity filter, guiding $Na^+$ ions towards the vestibule of the channel and repelling negatively charged ions. The M2-M3 loop is a crucial element in the transduction processes of the binding information leading to channel gating due to its strategic position, as confirmed by the presence of conserved residues in its sequence. Mutating spe-

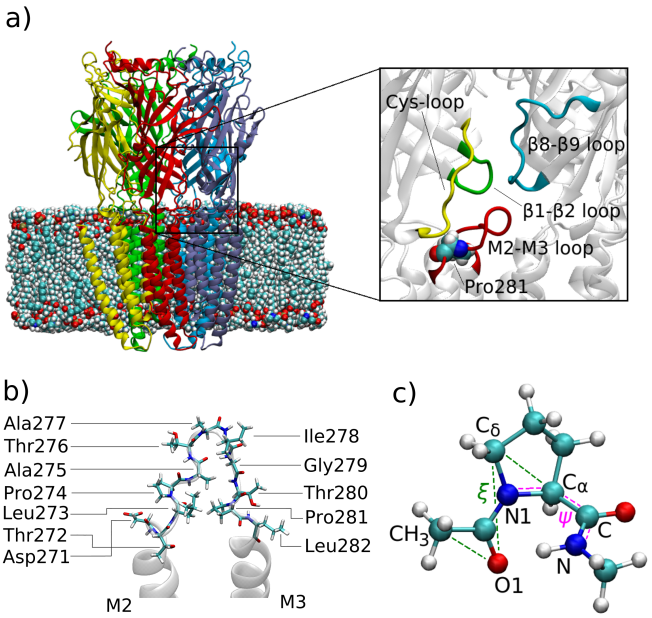



Figure 1: a) Section of the 5-$HT_3$R model used in this work, embedded in a POPC lipid bilayer. Subunit 1 is coloured in red, 2 in yellow, 3 in green, 4 in light blue and 5 in blue. A zoomed view of the ECD-TMD interface is shown, with the Cys-loop highlighted in yellow, the $\beta$1-$\beta$2 loop in green, the $\beta$8-$\beta$9 loop in blue and the M2-M3 loop in red; Pro281 is shown as van der Waals spheres. b) The residues of the M2-M3 loop. c) Proline dipeptide; the torsional angles $\xi$ and $\psi$ are shown in green and magenta respectively.

cific residues in the M2-M3 loop produced partially or non-functional channels, whose neurotransmitter binding properties were however untouched.[20] In particular, through unnatural amino acid mutagenesis, Pro8* (here Pro281) was substituted in a mouse 5-$HT_3$R with proline analogues that favour or disfavour the *cis* isomer to various degrees with respect to the natural ratio. Non-functional channels were produced when Pro281 was mutated to residues strongly favouring the *trans* conformer, suggesting that, without Pro281 *trans-cis* isomerization, the channel would not open.[20] A correlation between the *cis-trans* free energy difference of the tested proline analogues and the activity of the channels measured through the effective concentration half ($EC_{50}$) was observed among functional 5-$HT_3$ receptors and confirmed by free energy calculations of proline analogue dipeptides in aqueous solution.[21] These data led to the hypothesis that Pro281 *trans-cis* isomerization may act as a key molecular switch in the chain of structural rearrangements culminating in the opening of the ion channel, i.e. in perturbing the hydrophobic gate made of a five-member ring of leucines (Leu260, at position 9' in the M2 helix), which hinders the flux of ions through the TMD in the non-conductive state.[22] It was originally suggested[20] that the $\beta$1-$\beta$2 loop and the Cys-loop may pin Pro281 in the *trans* conformation, while agonist binding may displace the Cys-loop, thus releasing this residue.

The role of proline isomerization is however controversial, although it is recognized that this proline plays a significant role in channel function. For example, mutagenesis electrophysiology experiments on the homologous human 5-$HT_3$ receptor showed that the substitution of Pro8* with a histidine or a tryptophan did not significantly compromised channel gating by serotonin,[23] although they produced other effects such as a different dependence on the extracellular $Ca^{2+}$ concentration and an increase in the rate of desensitisation.[23] This was interpreted as a demonstration that proline isomerization is not necessary, although histidine and tryptophan may compensate for the absence of proline, e.g. through hydrogen bind-

ing, leading to functional channels. Moreover, the proline switch may not be a route of opening that applies to all pLGICs. In the nicotinic acetylcholine receptor (nAChR), for instance, the role of the equivalent Pro8*, investigated through mutagenesis experiments, was attributed to its steric and hydrophobic compatibility with the surrounding residues which allows it to be involved in the coupling between ligand-binding in channel gating rather than to isomerization.[24] There are also questions about time scales, given that proline isomerization may require long time in comparison to typical gating time of pLGICs, although the biochemical environment surrounding proline could affect the energetic barrier and speed up the process and different pLGICs have substantially different gating rates.[20,23,25,26] More experimental evidence is needed to establish unambiguously whether proline isomerization, which is emerging as a crucial component in a number of cell signalling cascades,[27] is or is not a critical step in the working mechanisms of pLGICs, and to assess its generality. Interesting and useful insights at the molecular level, not accessible to experiments, can be meanwhile provided by computer simulations.

In this work, we explore the *trans-cis* isomerization of Pro281 in the 5-$HT_3$ receptor with the specific goal to assess how such process is affected by and, in turn, affects the structural and electrostatic environment of the ion channel, using molecular dynamics and the enhanced sampling method metadynamics,[28–30] which has been successfully used to accelerate rare events and explore free energies in small and large biomolecules[31–33] and proline-containing systems.[21,34–38] Our receptor model is based on the 5-$HT_3$R X-ray structure by Hassaine et al.[8] and includes the full ECD and TMD, embedded in a lipid bilayer in a solvated environment. We carried out metadynamics calculations to estimate the free energy landscape of the isomerization, as well as a series of molecular dynamics simulations to assess the cumulative/cooperative action of multiple proline switches on the channel and, in particular, on the selectivity filter. Besides Pro281, close to the top of the M3 helix which was proved crucial for the function of 5-$HT_3$R,[20] we also investigated Pro274 which is close to the top of the M2 helix. Unlike Pro281 (which is only present in 5-$HT_3$R and nAChR), Pro274 is conserved across the Cys-loop superfamily,[9] and and may be involved in the gating mechanism of pLGICs through its interaction with the $\beta 1$-$\beta 2$ loop.[7,9,39–41] All the simulations were run with the NAMD 2.9 MD package,[42] the AMBER ff14SB[43] and LIPID14[44] force-fields, while the Plumed 2.0 plugin[45] was used for metadynamics. Details of the model, including a comparison with the newly available structures, and the simulation protocol are available in the Supplementary Information.

We first assessed how the protein environment affects proline behavior. In Fig. 2 the free energy surfaces (FES) at room temperature describing the isomerization process of a proline dipeptide in water and of Pro281 within the M2-M3 loop in 5-$HT_3$R are shown. The FES were calculated as a function of the torsional angles $\xi$ (torsion about the prolyl peptide bond linking Thr280 and Pro281) and $\psi$ (torsion about the bond that precedes the peptide bond linking Pro281 and Leu282; the peptide bond between Pro281 and Leu282 remains in its standard *trans* isomer). These angles are shown in the proline dipeptide model in Fig.1 c) and their reliability in properly describing proline isomerization has been previously demonstrated.[21,34,46] The transition from

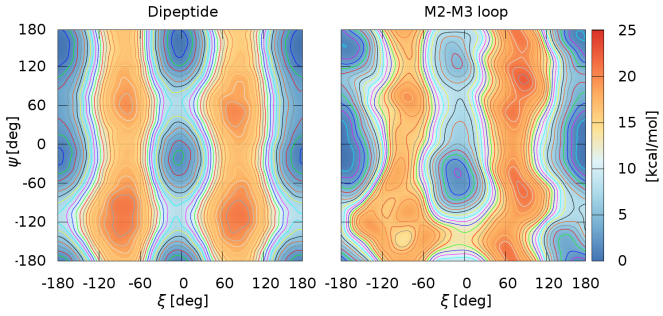



Figure 2: Free energy surfaces of proline dipeptide in water (left) and of Pro281 in 5-$HT_3$R (right) as a function of the torsional angles $\xi$ and $\psi$. The absolute minimum has been set to zero and contour lines are every kcal/mol.

the simple dipeptide in solution to the protein environment, within a flexible loop in contact

with lipids and numerous sources of interactions from different directions, makes the exploration and convergence of this map more challenging, as manifested by the factor of 20 in the sampling time needed for convergence (40 ns vs 800 ns). The effects of the biomolecular environment are also reflected in the more rugged and less symmetric FES in 5-$HT_3$R, although four basins of minimal free energy can still be identified; the energy barriers are increased, reaching 25 kcal/mol in the 5-$HT_3$R map, while they never overcome 20 kcal/mol in the dipeptide. The transition path for the $\xi$ *trans* to *cis* isomerization is more likely to be counterclockwise in 5-$HT_3$R with higher barriers at $\xi \sim 90°$ than $\sim -90°$, while in the dipeptide clockwise and counterclockwise transition paths have similar free energies. In 5-$HT_3$R, the favoured *trans-cis* isomerization path implies that the Pro281 cyclic side chain and the Thr280 side chain move under the M2-M3 loop. This may be sterically enforced by the presence of the Leu282 side chain oriented outward at the top of the M2 helix and by the aromatic ring of Tyr140 in the Cys-loop, which comes into contact with the M2-M3 loop from the ECD and may hinder the rotation in the opposite direction.

The free energy surface obtained from metadynamics simulations does not directly provide information on the isomerization kinetics and the transition rates between basins. We observed a path selection rather than a barrier reduction with respect to the simple proline dipeptide model. However, forcing a preferential rotational path may affect the interactions within the M2-M3 loop area, and modulate the transduction of mechanical signals through to the ECD-TMD interface.

The *trans* and *cis* Pro281 isomers are characterized by the different orientation of the backbone oxygen of Thr280 (O1 in Fig. 1 c) ), which can act as acceptor of hydrogen bonds; Thr280 hydroxyl group and amino group can also participate in hydrogen bonds. Fig. 3 a) shows a block average of the number of hydrogen bonds formed by Thr280 within 5-$HT_3$R as a function of the isomerization angle $\xi$, over the metadynamics simulation time, providing qualitative information on potential interactions at different values of $\xi$.

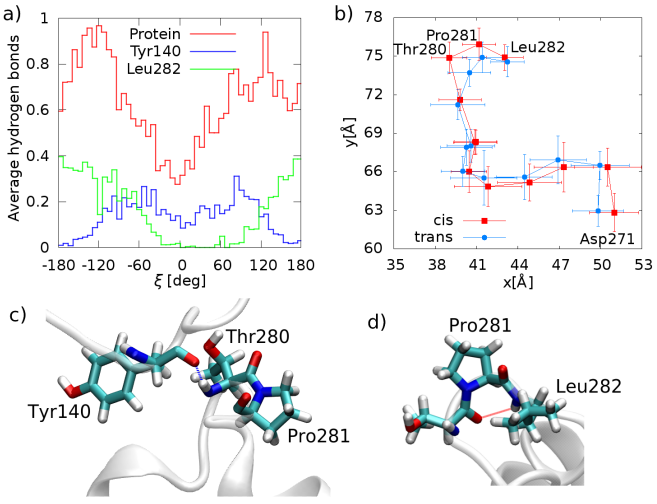



Figure 3: a) Average number of hydrogen bonds formed by Thr280 during the metadynamics simulation with other protein residues (red), Tyr140 in the Cys-loop (blue), and Leu282 in the M2-M3 loop (green). b) Average positions of the M2-M3 loop residues occupied in the plane orthogonal to the ion channel of the $C_\alpha$ during the metadynamics run. Pro281 is shown in *cis* (red) and in *trans* (blue). c) and d) Exemplary configurations and interactions for the *cis* and *trans* Pro281 isomers respectively.

The average number of hydrogen bonds between Thr280 and other protein residues has its maximum values around the $\xi$ transition states; their main contributions arise from the hydrogen bond between Thr280 and the Cys-loop Tyr140 (observed in the transition states and in the *cis* isomer, but not in the *trans*), and the hydrogen bond between O1 and the NH group of Leu282 at the top of the M3 helix in the *trans* isomer, also observed in the dipeptide model. Exemplary configurations highlighting these bonds are shown in Fig. 3.

The hydrogen bond with Tyr140 is particularly interesting because this residue is likely to be part of the transduction pathway from the extra-cellular to the transmembrane domain. In a neurotransmitter-bounded receptor, the relative position of the Cys-loop and the M2-M3 loop may be perturbed and shift the competition between the mutually exclusive hydro-

gen bonds involving Thr280 and either Leu282 or Tyr140 in favor of the latter, encouraging isomerization. In an alternative scenario, the distance between Thr280 and Tyr140 may increase, removing the steric hindrance and lowering the isomerization barrier. The 5-$HT_3$R X-ray structure does not however include ligands in the orthosteric sites and in this study we did not take their effects into account as the timescale to propagate the signal would likely be too long for MD-based simulations; biasing it with metadynamics would not be an obvious process. Hence, we chose to focus on the effects on proline isomerization within the given biomolecular environment.

The average positions and corresponding standard deviations of the $C_\alpha$ of the residues of the M2-M3 loop in the plane orthogonal to the ion channel ($x$-$y$) were calculated for the configurations of the *trans* isomer ($-180° < \xi < -150°$; $150° < \xi < 180°$) and the *cis* isomer ($-30° < \xi < 30°$) and are shown in Fig. 3 b). As expected, the main variations are localized around the isomerization site, in particular near Pro281 and Thr280, and, surprisingly, at the site of the negatively charged Asp271 at the top of the M2 helix, which acts as a selectivity filter to attract the $Na^+$ ions. This possible correlation between Pro281 isomerization and Asp271 at opposite edges of the M2-M3 loop is interesting, although still relatively small with respect to the standard deviation for a single isomerization event.

The metadynamics simulations were limited to a single proline isomerization within one subunits and did not account for potential cooperative effects in the pentameric receptor. To explore this possibility, and the more general impact of proline isomerization on the protein environment, we performed 500 ns unbiased MD simulations at room temperature for 5-$HT_3$R models where three non-consecutive (3Cis) and all five (5Cis) Pro281 were first forcefully isomerized by means of metadynamics, and compared the results against a model with all Pro281 in the original *trans* isomer (5Trans), and an additional model where all five Pro274 were isomerized to *cis* while all five Pro281 remained in the *trans* (5Trans5Cis) configuration. The goal here was to capture mechanical effects in the surroundings of the M2-M3 loop where the isomerization had taken place. Once the switches were induced with metadynamics, the *cis* isomers of Pro281 (or Pro274) were naturally maintained during the entire length of the MD simulations without the need of any restraining bias, in accordance to what observed in the FES previously discussed. Moreover, we did not restrict the access to the two basins in $\psi$ since they are separated by lower barriers than those associated to the $\xi$ *trans-cis* isomerization. The M2-M3 loop were rather flexible during the MD similations with an average root mean square fluctuation with respect to the equilibrated structure equal to 1.9 Å for 5Trans, 3Cis and 5Trans5Cis and 2.4 Å for 5Cis, which shows the larger fluctuations.

The average ion channel width along the channel axis, with the zero set at the hydrophobic gate, is shown in Fig. 4. While the result obtained with the 5Trans model confirms previous simulations on a model based on the same X-ray structure,[19] upon isomerization of Pro281, the upper part of the channel (between 15 and 20 Å in Fig. 3) undergoes (from 3Cis to 5Cis) a gradual restriction of the selectivity filter radius, with larger fluctuations in the same quantity. This restriction is slightly more marked in the 5Trans5Cis case. No change is instead observed at the level of the hydrophobic gate and the channel remained in a closed state in all models within the sampled time.

The distributions of the Asp271 $C_\alpha$ positions in the $x$-$y$ plane orthogonal to the channel axis in the five subunits of each model are shown in Fig. 5 a), together with the respective averages and standard deviations. In 5Cis, 3Cis and 5Trans5Cis rearrangements of Asp271 led to a significant reduction of the area of the corresponding pentagons, which became more symmetric and gave rise to the effect seen at the selectivity filter level in the channel width graphs; these structural rearrangements are consistent with the qualitative indication from metadynamics reported in Fig. 3 b). No significant changes in the position of Leu260 at the hydrophobic gate were observed.

The constriction of the upper part of the pore

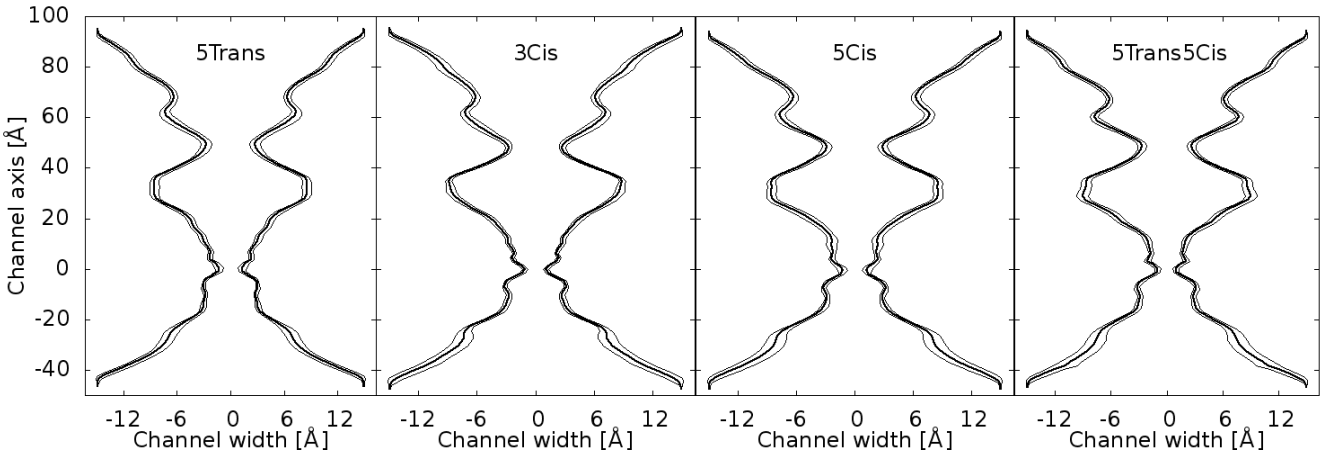



Figure 4: Average channel width profiles of (from left to right) the 5Trans, 3Cis, 5Cis and 5Trans5Cis models in the MD simulations. The zero of the channel axis is set at the height of the hydrophobic gate (Leu260).

containing the ring of negative Asp271 may enhance the attraction of $Na^+$ ions towards the vestibule of the channel. However, this constriction cannot be too strong otherwise ions would not be able to pass through. Hence, the effect produced by Pro281 isomerization may be just optimally tuned for this to happen, compared to what the isomerization of Pro274 would imply. Interestingly, the newly determined cryo-EM conductive state structure in Ref.[15] shows a constriction in the width of the channel at the level of Asp271, which might be a signature of the effect on the Asp271 ring observed in our simulations, although all Pro8* in the cryo-EM models are in the conventional *trans* isomer.[14–16]

To further quantify the effect of the Asp271 arrangements in the different models, the average electrostatic potential of the five Asp271 was measured; a representative section of the resulting maps at the height of the selectivity filter is represented in Fig. 5 b). Upon the isomerization of Pro281 the electrostatic potential minimum within the five Asp271 decreases considerably and shifts towards subunit 3 and 4. On the other hand, the isomerization of Pro274 does not seem to affect considerably the map, while slightly increasing the minimum.

In summary, we used molecular dynamics and metadynamics to investigate at the molecular level how a *trans-cis* proline molecular switch, potentially involved in the working mechanism of the 5-$HT_3$ pLGIC, is affected by the complex protein environment, which it affects in turn. Within the complex receptor, the isomerization free energy landscape loses the symmetry typical of proline dipeptide in aqueous solution, acquiring a favourable counterclockwise rotation pathway through steric hindrance and interactions with neighbouring residues. An important role in transmitting the signal from the ECD upon neurotransmitter binding may be played by the Cys-loop, e.g. through Tyr140 interacting with the M2-M3 loop Thr280 in the *trans-cis* transition state or, alternatively, through its side chain moving out of the way. We can speculate that such effects on the interface loops may be selected and amplified by the perturbation induced by neurotransmitter binding. A relative twisting of the ECD with respect to the TMD, as suggested for other pLGICs,[39] may indeed modify the network of contacts between the residues of the M2-M3 loop and those of the Cys-loop and other interface loops, affecting the isomerization free energy barrier, the stability of *cis* isomer and the rate of isomerization. Additional effects due to the pLGIC-lipid interaction have not been treated here, but are worth investigating further.[47]

Our simulations show that proline isomerization in the M2-M3 loop transmits a subtle but consistent mechanical effect to Asp271, which acts as an attractor for the $Na^+$ ions at the top of the M2 helix. Different numbers and

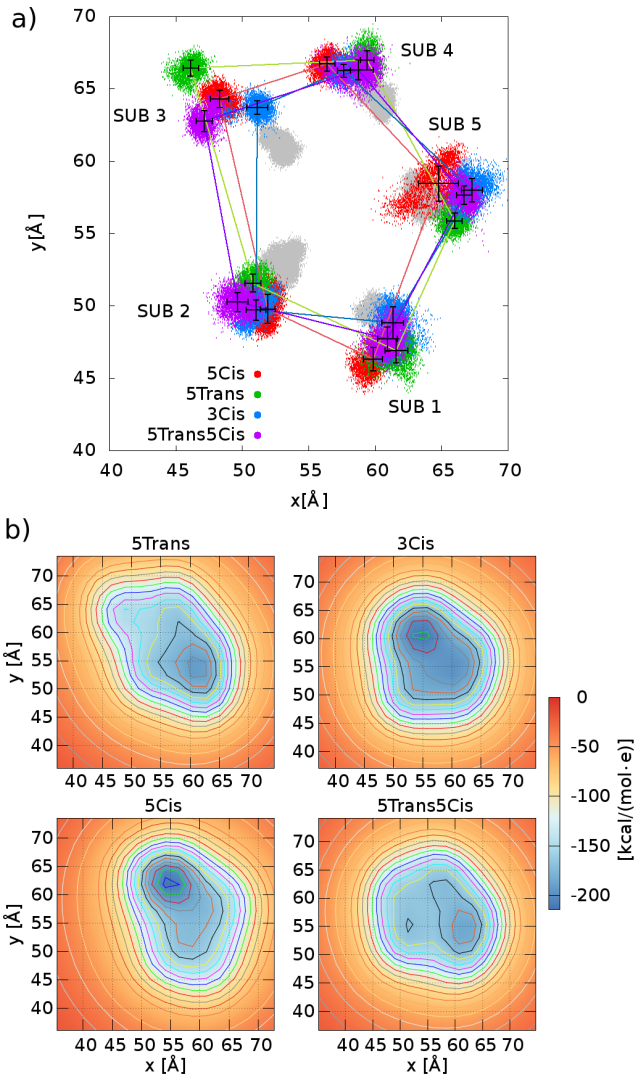



Figure 5: a) Positions in the plane orthogonal to the ion channel of Asp271 (including average and standard deviation, coloured according to the model) and of Leu260 (in grey for all models) in the MD simulations. b) Maps of the electrostatic potential generated by the Asp271 ring in: 5Trans (top left), 3Cis (top right), 5Cis (bottom left), and 5Trans5Cis (bottom right).

sequence of isomerization events in the subunits may help modulate to different degrees the radius and shape of the Asp271 charge-selective ring, pulling the ions towards the channel vestibule, so to be ready to cross the hydrophobic gate once this is enlarged in a subsequent step. It has been proposed that the latter may involve both Leu260 and Val264.[33]

While a full account of the chain of events triggered by neurotransmitter binding and resulting in channel gating in 5-$HT_3$R is still far from be revealed, our work provides interesting molecular-level insights into the mechanical and electrostatic effects of the experimentally suggested *trans-cis* proline switch within the complex biomolecular environment. The very recent availability of new structures in a variety of functional states[14–16] opens the way for further studies.

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The data supporting this research will be made openly available at the King's College London research data archive. The Supplementary Information includes details of the model and the simulation protocol.

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